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SUMMARY OF NITROSAMINE RESEARCH AT THE EASTERN REGIONAL RESEARCH CENTER

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The Eastern Regional Research Center (ERRC), Meat Laboratory initiated studies on nitrosamines (NA's) in cured meat products in 1970. This was shortly after the U.S. Food and Drug Administration (FDA) found dimethylnitrosamine (DMNA) in fish products treated with NO_2^- and/or NO_3^- (1).

Initially, commercial samples of ham were analyzed for DMNA, but none was detected (2). In a survey of frankfurters, 11 to 84 ppb (μg/kg) DMNA was confirmed in 3 of 40 samples analyzed (3). Therefore, two approaches were taken to determine the cause of DMNA formation: to determine chemically whether some biogenic amines had any role in DMNA formation, and to determine the effect of cure ingredients on DMNA formation in processed frankfurters.

In the first study, tertiary amines containing a dimethylamino group were found to produce a greater amount of DMNA than the corresponding quaternary ammonium compounds. These results suggested that tertiary amines, under certain conditions, may pose as great a problem in NA formation as secondary amines (4). Secondly, in processed frankfurters, a concentration of 10 ppb or greater DMNA could not be produced when franks were prepared with the permitted level of ca. 150 ppm (mg/kg) $NaNO_2$. Ten times this amount was needed to consistently yield 10 ppb DMNA (5) which was the lowest concentration of NA confirmable by mass spectrometry at that time. It was found that added $NaNO_3$ had little effect on DMNA formation (6), but sodium ascorbate (NaAsc) and sodium erythorbate (NaEry) inhibited its formation (7). At the same time we carried out this work it was also reported that ascorbic acid (AscH) inhibited NA formation from drugs (8). Cure ingredients in a frankfurter model system were also investigated. Generally, AscH, NaAsc, and NaEry were found to inhibit the nitrosation of dimethylamine, while glucono-delta-lactone (GDL) was found to enhance DMNA formation (9). Preliminary experiments with GDL in frankfurters also showed the same effect.

The study on frankfurter cure was not completed because reports by other investigators of the consistent occurrence of nitrosopyrrolidine (NPy) in fried bacon (10,11,12) raised questions of higher priority. Since the consumption of bacon exceeds 1.5 billion pounds annually in the United States, this problem required immediate attention.

The questions that needed to be answered were: how is NPy formed and how do you prevent its formation? Nitrosopyrrolidine may result from the nitrosation of pyrrolidine (Py) derived from putrescine or from the decarboxylation of proline (Pro) or by the formation of nitrosoproline (NPro) and its subsequent decarboxylation. Kinetic data by Mirvish (13) suggests that Pro, a weaker base than Py, would nitrosate more readily. The fact that NPy is not present in uncooked bacon (11,12,14) provides indirect evidence that formation occurs via the NPro pathway. If this is correct, it would theoretically be possible to prevent conversion of NPro to NPy.

Heating NPro in silicone oil in a model system at different temperatures showed that maximum production of NPy occurs at 185°C (365°F), close to the recommended bacon frying temperature, and that little is formed at lower temperatures (14). Therefore, the frying conditions might be important in determining how much NPy is formed in the cooked product.

Two experiments: 1) frying bacon to medium-well done using 4 different temperatures from 99° to 204°C and varying time; and 2) holding the frying time constant at the same temperatures with the degree of bacon crispness variable; showed that NPy formation was primarily temperature dependent. Lower temperatures and longer frying times yielded less NPy (14). Other commonly used cooking methods were also investigated. When bacon was cooked by frying, baking, broiling, using a microwave oven, or an appliance called a "baconer", the least amount of NPy was found in samples cooked by the microwave oven (14).

Next, investigations on bacon storage and processing were conducted to determine their contribution to NPy formation. Vacuum packages of freshly prepared bacon stored at 7°C for up to 10 weeks prior to frying showed a slight decrease in NPy formation (15). Bellies fried and analyzed after cure injection, prior to introduction into the smokehouse, and after the drying step contained ca. 8 ppb NPy. A significant increase in NPy resulted from the remaining heat and smoking treatment (16).

These results suggested that processing time may be an important factor in NPy production since it varies from one manufacturer to another. However, repeated processing of bacon up to 24 hours with sampling at 4-hour intervals showed no clear-cut correlation between NPy concentration and processing time (17).

Investigations were also carried out on "green" uncured bellies. Storage of green bellies under refrigeration conditions up to 2 weeks resulted in no change in either free Pro prior to processing or NPy in the finished bacon (16). However, a significant increase in free Pro was observed in green bellies frozen for 2 weeks and then thawed. A corresponding change in NPy content was observed after processing the bellies into bacon. This finding was clear-cut only when bacon was made without NaAsc or NaEry (18).

Earlier investigations showed higher concentrations of NPy in the cooked-out fat than in fried bacon (11,12). This may result from NPy partitioning into the fat phase due to its fat solubility. When separated bacon adipose and lean tissue were subjected to similar frying conditions, NPy was shown to form only from the adipose tissue (19). Assuming NPro is the precursor for NPy, why doesn't ham also form NPy since uncured ham contains an average of 52 ppm Pro (20) as compared to 17 ppm in green intact bellies (18)? The unique composition of pork belly adipose tissue may explain why ham, Canadian bacon (back bacon), and a beef bacon-like product do not form NPy under similar frying conditions (19). It appears that NPy formation is complex and it is theorized that connective tissue or collagen, known to be composed of large concentrations of bound glycine, hydroxyproline and proline, is directly or indirectly responsible. At this time, the role of NPro is not clear since we have isolated and identified it in uncooked bacon in concentrations that could yield NPy at the ppb level after frying (21).

Since NPy is formed primarily in the adipose tissue of bacon, the NA inhibitory activity of NaAsc and NaEry may be limited by their low fat solubility. Therefore, a number of more fat soluble derivatives of ascorbic acid, in combination with NaAsc, were evaluated for inhibition of the nitrosation of Py in a bacon model system. Ascorbyl palmitate and dipotassium ascorbyl-2-sulfate were particularly effective (22), and are currently being investigated for their NPy inhibitory activity in bacon. These studies are being conducted because earlier work in our laboratory and those sponsored by the American Meat Institute (AMI) failed to eliminate NPy in bacon prepared with 1000 ppm NaAsc or NaEry. This approach of interfering with the nitrosation reaction by reducing the NO_2^- to nitric oxide, a compound that does not normally nitrosate secondary amines, has to date not been completely successful. The reductant competes with amines for NO_2^- and is effective if the rate of reaction is greater. Reducing added NO_2^- is an attractive approach to decreasing NA's because their formation is generally dependent on the square of the NO_2^- concentration. However, little information is available as to how much the NO_2^- can be lowered without having problems with flavor, color, and more importantly, microbiological preservation, particularly against C. botulinum. Our laboratory has been interested in two alternatives that would control C. botulinum and still permit use of lower levels of NO_2^-. The first uses the irradiation sterilization of bacon. This is an extension of our work on ham in collaboration with the U.S. Army Natick Laboratory (23,24). The other approach, in cooperation with the AMI, utilizes prefried bacon. Here, C. botulinum is controlled by reduced water activity and high concentration of salt resulting from the removal of rendered fat and moisture during the brief prefrying process (25). This work has not been completed.

Several other investigations have been carried out. Recognizing that gelatin is commonly prepared by the alkaline treatment of collagen, gelatin could serve as a source of nitrosatable proline that may be converted to NPy under certain conditions. Therefore, a limited volatile NA survey was carried out on meat products made with gelatin and chunks of cured nonskeletal and organ meats. DMNA was confirmed in 8 of 10 samples of souse, head cheese and blood and tongue loaf, but only a single sample containing NPy. No correlation was found between residual NO_2^- and the concentration of DMNA (26).

In a study of lecithin and its derivatives, the results suggest that phospholipid decomposition products may be a source of DMNA (27). The lecithin content of nonskeletal tissue, including organ tissue, may be higher than skeletal tissue. In fact, phospholipids have been found in bovine tongue (28) and the same is probably true of porcine tongue, which is a cured ingredient of souse.

Some research has been conducted on fermented meat products, specifically with Lebanon bologna, an all beef product, and with pepperoni in cooperation with our Meat Microbiology group. NA's could possibly be formed in this product type as a result of its pH, NO_3^- cure and the microorganisms present. None of the volatile NA's surveyed were found in commercial samples of Lebanon bologna or in those prepared in our pilot plant with natural meat flora or starter culture during the fermentation and mellowing period (29). A similar study of pepperoni prepared in our laboratory also gave negative NA results (30). These studies suggest that volatile NA's were not formed and decomposed during processing and that NA's in fermented meats may pose little or no problem.

In cooperation with the Plant Products Laboratory, samples of fresh and processed spinach and beets originally containing 1500 to 2000 ppm NO_3^-, which had been subjected to storage abuse, were analyzed for NO_2^- and NA's. No NA's were detected, even in samples stored beyond the point of edibility (31).

Although we have investigated other areas, NPy in fried bacon remains our priority project.

This summary would not be complete without some mention of the analytical procedures employed. The difficulty in the NA analysis has been found to be related to the fat content of the meat product. That is, the complexity and amount of sample clean-up required increased as our analyses progressed from ham to frankfurters, then fried bacon, and finally its cooked-out fat. Sampling, specifically with bacon, is a persistent problem due to its inherent variability. Procedures for the isolation and determination of volatile NA's developed by the U.S. FDA are used for the analysis of meat samples (32,33,34). We have modified them slightly as required. The column chromatographic clean-up step is very critical. The silica gel must have the proper activity in order to obtain a relatively clean sample and good NA recovery so that it can be quantitatively determined and confirmed. The samples are assayed for six volatile NA's which include: dimethylnitrosamine, methylethylnitrosamine, diethylnitrosamine, nitrosopiperidine, nitrosopyrrolidine, and nitrosomorpholine. Separation and detection is carried out with a gas chromatograph equipped with a column containing Carbowax 20M-TPA liquid phase and a nitrogen selective alkali flame ionization detection (AFID). In our initial work with ham, a Varian-Aerograph rubidium sulfate pellet detector was used. However, problems were encountered with reproducibility of response toward NA's. A potassium chloride coated Pt-Ir wire coil AFID is currently used. The detector response to NA's is linear over a wide range and 0.1 ng NA is readily detected. Occasionally, sample peaks, which are not NA's, are obtained at the same retention time as NA's. Confirmation of identity of NA's is carried out by gas-liquid chromatography-mass spectrometry (glc-ms). The mass spectrometer is operated in the peak matching mode at a resolution of 1 in 12,000. A glc retention time coincident to an authentic NA and matching the exact mass of the NA parent ion are the criteria for positive confirmation. For example, the parent ion of NPy is m/e 100.06366. Concentrations as low as 3 ppb have been confirmed with this single ion detection technique depending on the amount of interfering sample components present; 5 ppb NPy or higher are confirmed with more consistency.

Some of the same techniques have been applied to the isolation and identification of NPro in uncooked bacon. The volatile methyl ester was used for gas-liquid chromatographic detection and mass spectral confirmation (21). However, a reliable quantitative procedure for NPro has not been developed. Currently, high-pressure liquid chromatography is being used in an attempt to overcome the isolation problems.

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Precaution should be exercised in the handling of nitrosamines since they are potential carcinogens.